Commentary on the recent FSH collection: known knowns and known unknowns.

Short title: FSH in reproduction and beyond

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Abstract

Follicle-stimulating hormone (FSH) is a dimeric glycoprotein secreted by the anterior pituitary gonadotrope, which is necessary for reproductive function in mammals. FSH primarily regulates granulosa cells and follicular growth in females, and Sertoli cell function in males. Since its identification in 1930s and sequencing in 1970s, significant progress has been made in elucidating its regulation and downstream function. Recent advances provide deeper insight into FSH synthesis and effects in the gonads, suggest potential roles in extragonadal tissues, and examine pharmacological approaches and clinical applications in infertility treatment, that now affects 18% of couples. These advances were discussed in detail in a number of reviews published in the last two years in *Endocrinology*. In this brief commentary, we summarize these reviews and point to the outstanding questions that should be answered in the near future to bridge a gap in our understanding of this hormone.

Follicle-stimulating hormone (FSH) is a gonadotropin hormone critical for reproductive function. It is synthesized and secreted from the anterior pituitary gonadotrope cells, primarily under the influence of hypothalamic GnRH neuropeptide, and ovarian or locally produced activin-inhibin-follistatin system (reviewed recently in (1)). Structurally, FSH is a dimeric glycoprotein composed of a unique β subunit and a common α subunit shared with luteinizing hormone (LH) and thyroid-stimulating hormone. Contrary to LH, FSH secretion is not entirely regulated, and most of FSH is constitutively released. FSH in males stimulates Sertoli cell proliferation during development and maintains Sertoli cell function in adults, by inducing androgen binding protein that contributes to spermatogenesis through interaction with testosterone. In females, FSH stimulates growth of ovarian follicles and aromatase expression in follicle granulosa cells, which synthesizes estrogen. FSH deficiency in humans results in the absent or incomplete pubertal development in women, relatively normal pubertal development but azoospermia in men, and infertility in both women and men. Female mice deficient in FSH have a block in folliculogenesis prior to the antral stage resulting in infertility, while males have impaired reproductive function due to lower sperm count, but are not infertile (variety of FSH mouse models reviewed recently in (2, 3)).

FSH concentration displays two temporally separate increases during female reproductive cycle, as opposed to LH which exhibits a single rise. During the rodent estrous cycle, a surge of GnRH during the afternoon of proestrus triggers a surge in both LH and FSH, resulting in ovulation of the mature follicle in response to LH. Several hours later, during the morning of estrus, a secondary FSH increase occurs, without a corresponding rise in LH. This secondary FSH rise is essential for follicular development for the subsequent cycle in rodents. In humans, FSH level increases in the late luteal phase through the mid-follicular phase of the menstrual cycle, in addition

to the preovulatory rise, corresponding to the recruitment of a new cohort of follicles to the growing pool. During reproductive aging in females, rise in FSH is considered the hallmark of the reduction in the follicle reserve. The fall in inhibin B caused by the loss of follicles, coincides with the rise in FSH, supporting the primary role of inhibins in the negative feedback of FSH.

Normal physiological function of FSH in women follows Goldilocks principle: low FSH impedes follicular growth, while high levels are associated with premature ovarian failure. Mouse models of FSH overexpression exhibit female infertility of unknown ovarian etiology, with follicles at various stages of development, lack of corpora lutea and the presence of large hemorrhagic and fluid-filled cysts (3). Premature ovarian failure (POF) is a disorder affecting 1% of reproductive age women that lose ovarian function before the age of 40, 70% of which are of idiopathic causes. POF can occur due to an accelerated loss of follicles, an inability of the remaining follicles to respond to ovulatory signals, an initially diminished ovarian reserve, or any combination thereof. Although it is possible that high FSH in POF is solely a consequence of reduced ovarian inhibin level, high FSH from any cause can potentially lead to the recruitment of a larger number of follicles into a growing pool in every cycle in younger women resulting in early depletion (1, 4). In fact, increased levels of FSH in the circulation, due to re-trafficking of FSH to the GnRH-regulated secretory pathway or to ectopic overexpression, resulted in significantly more corpora lutea, implicating higher FSH levels in greater follicle recruitment in these mouse models (3). Therefore, studies addressed in recent reviews all demonstrate that proper regulation of FSH concentration and function is critical for female fertility. In this brief commentary, we will summarize recent reviews published in *Endocrinology*, discussing FSH synthesis and function, and point to outstanding questions, answers to which will provide further insight into the physiology and pathophysiology of the reproductive system.

Transcriptional regulation

The unique FSH β-subunit provides biological specificity and is the limiting factor in the mature FSH synthesis, since α-subunit (αGSU) is minimally regulated by hormones and is expressed at the sufficiently high basal level. While concentration of LH in the circulation is regulated at both the level of β-subunit transcription and GnRH-stimulated secretion, FSH concentration is regulated primarily at the level of β-subunit transcription, since FSH is constitutively secreted. FSH increases together with LH during the preovulatory surge and exhibits a separate, second increase that is necessary for folliculogenesis in humans and rodents. Changes in FSHβ mRNA (*Fshb*) levels precede changes in FSH concentration in the circulation. Transcription of *Fshb* is induced primarily by activin and GnRH. In mice lacking GnRH or GnRH receptor, FSH levels, as well as LH levels, are 60%-90% lower in males and females. GnRH injection in rats with low endogenous GnRH increased FSHβ transcription 4-fold, comparable to the changes in FSH concentration in the circulation throughout the cycle. Signaling pathways and molecular mechanisms of this induction are reviewed by Stamatiades, et al., (5). *Ex vivo* studies using primary cells or studies using gonadotrope-derived model cell lines determined roles for calcium-activated pathways, calcineurin and CamKII, as well as for ERK1/2 MAPK and PKA pathways, in *Fshb* induction by GnRH. These pathways activate AP-1 or CREB transcription factors that replace repressors JDP2 or ICER on the *Fshb* promoter to activate *Fshb* transcription. Although whole body null mice of several of these players confirm their roles in maintaining FSH levels, studies using gonadotrope-specific deletions are either lacking or do not support findings *in vitro*. Future studies are needed to accurately determine the roles of identified signaling molecules and transcription factors *in vivo* and to establish mechanisms of GnRH regulation.

Activin is a potent regulator of FSHβ gene expression and was originally identified as a component of ovarian follicular fluid that increased FSHβ synthesis and FSH secretion from pituitary gonadotrope cells. Expression of both intrapituitary follistatin and ovarian inhibin, inhibitors of activin, fluctuate during the estrous cycle in opposition to the levels of FSHβ mRNA, suggesting that bioavailability of activin, through changes in follistatin and/or inhibin levels, is a critical regulatory component of FSHβ synthesis. In particular, the secondary rise of FSH that is necessary for follicular development is dependent on activin since, both FSHβ mRNA and FSH levels in the blood can be reduced during the secondary rise in female rats infused with follistatin. *Fshb* regulation by activin / TGFβ family of proteins was reviewed by Fortin, et al., (6) and Ongaro, et al., (7).

As with GnRH, studies using primary pituitary cells *ex vivo* or cell lines demonstrated that activins activate *Fshb* expression through a canonical type I and II receptors and Smad pathway. Smad3 is phosphorylated by the type I receptor, ALK4 primarily, dimerizes with Smad4, and translocates to the nucleus where it binds DNA either directly or via its interactions with Smad4 and/or Foxl2 forkhead factor. Smad2, which can also be phosphorylated by activin signaling, is not critical for *Fshb* induction by activin. Although Smad3 links activin receptor to transcriptional activation, pituitary *Fshb* expression and fertility are unaffected in mice with a targeted deletion of Smad3 in gonadotropes, either alone or in combination with Smad2. Since a truncated Smad3 protein that lacks the DNA binding domain is expressed in the gonadotrope following gonadotrope-specific Cre cross, Smad3 may regulate *Fshb* transcription without direct DNA binding. Mice with Smad4 deletion in gonadotropes, on the other hand, are hypogonadal and females are subfertile with impaired ovarian follicle development. Both male and female Smad4 conditional knockout mice exhibit diminished FSH levels and pituitary *Fshb* mRNA expression.

Deletion of Foxl2 in gonadotropes using a Cre/lox approach also causes reductions in *Fshb* and subfertility, with females producing smaller and fewer litters. The reproductive phenotype of a double Smad4/Foxl2 conditional knockout mice is more dramatic and resembles FSHβ knockouts: females are infertile and lack estrous cyclicity, with arrested ovarian follicle development at an early antral stage. Their FSH deficiency is also more pronounced than in the single knockout mice. The same signaling pathway may be shared with BMP subfamily, which stimulates *Fshb* expression *in vitro*. In particular, a role for BMP2, BMP4, BMP6 and BMP7 was proposed using cell lines (reviewed in (7)). Although specific receptors for BMPs, ALK1, ALK2, ALK3, and ALK6, most often phosphorylate Smad1/5/8 instead of Smad2/3, Smad4 is a common dimerization partner. Roles of ALK2 and ALK3 have been examined with gonadotrope specific deletion and results demonstrate that they are dispensable for *Fshb* expression. Since ALK1 and ALK6 expression is limited in gonadotropes, these results brought into question a role for BMPs in FSH regulation. In conclusion, studies demonstrate the necessity for Smad4 and Foxl2 is *Fshb* expression, however the roles of receptors (except for ALK2 and ALK3) and specific ligands, belonging to activin / TGFβ family, have not been determined. The variable effects of different ligands on *Fshb* may be a result of the complex interplay and crosstalk with the variety of type I and type II receptors.

Post-transcriptional regulation, translation, glycosylation and secretion

With an exception of glycosylation (reviewed in (8)) little is known about post-translational regulation, sorting and secretion of FSH. A couple of studies identified several micro-RNAs, miR-132 and miR-212 in particular, that regulate GnRH-stimulated FSH synthesis and secretion

(reviewed in (5)). miR-125b, on the contrary, prevents GnRH-induced FSH synthesis and serves as a repressor. Importantly, *in vivo* studies are remaining to confirm their roles. Further, potential roles of other non-coding RNA species regulating FSH levels need to be addressed. Additionally, translational control of two other gonadotrope-specific genes, *Lhb* and *Gnrhr*, is beginning to emerge (reader is referred to the recent commentary by MacNicol, et al., (9)), but it is completely unexplored in regards to *Fshb*.

FSH is an N-linked glycosylated protein, containing four N-acetylglucosamine residues (2 on each subunit) linked to an amide group of an asparagine (Asn) amino acid in the peptide chain (reviewed in (8)). Thus, FSH exhibits macroheterogeneity, resulting from the absence of one or more of the N-glycans; and microheterogeneity, reflecting different branching of glycans and variable sialic acid content, resulting in size and charge variants. As opposed to LH, where sulfated glycans are more abundant, sialic acid predominates in FSH, although sulfate and phosphate may provide alternative negatively charged residues. Glycosylation is critical for bioactivity, due to the necessity of glycosylation for dimerization of α and β subunits, subsequent glycohormone secretion, regulation of half-life in the circulation and binding to its receptor in the gonads. In particular, mutation of either one of two FSHβ N-glycans significantly increases clearance and reduces *in vivo* biological activity, whereas mutation of both sites eliminates biological function due to dramatically accelerated clearance. FSH glycosylation changes during puberty, reproductive cycles and aging, implying hormonal regulation of glycosylation. Accordingly, the biological activity of circulating FSH can vary under changing physiological conditions. Interestingly, during reproductive ageing in women, ratio of partially glycosylated, lower molecular weight $FSH^{21/18}$ to fully glycosylated FSH^{24} decreases. Several studies demonstrated that hypoglycosylated $FSH^{21/18}$ is more potent in activating FSH signaling pathway than FSH^{24}

(reviewed in (2) and (10)). The mechanisms underlying carbohydrate modulation of FSH activity are poorly understood. Although advances in mass spectrometry permit characterization of FSH glycan populations, positions, abundance, or linkages remain difficult to assess. However, understanding glycohormone regulation may be critical, since variants affect the biological activity of FSH, and glycosylations may vary in recombinant FSH preparations that are used in the clinic.

FSH is largely released constitutively, while LH is released in a pulsatile manner, via a regulated secretory pathway following GnRH stimulation. LH contains a carboxy terminal heptapeptide that directs its secretion via the regulated pathway. LH and FSH appear to diverge early in the secretory pathway, rather than later in the trans-Golgi network, where most of protein sorting occurs. For that reason, FSH only granules are relatively rare in the pituitary, while LH-only dense core granules can be observed, although majority of secretory granules contain both gonadotropins. However, it is still not clear if FSH associates with any chaperone or vesicular proteins in order to be sorted to the constitutive pathway. Questions also remain if there is any regulation of FSH secretion by hormones, such as steroids or activins, separately from their effects on β-subunit transcription.

Receptor binding and signaling

FSH binds and activates a 7-transmembrane domain, G-protein coupled receptor (FSHR) expressed in granulosa cells in ovaries and Sertoli cells in testes (reviewed in Ulloa-Aguirre, et al., (10) and Law, et al., (11)). In the granulosa cells, FSH regulates the expression of about 3800 genes that mediate proliferation and differentiation, and control the folliculogenesis from the preantral to the preovulatory stage. Such diverse functions are accomplished with complicated signaling pathways that are still not completely elucidated. FSHR can interact, directly or indirectly, with

other receptors such as IGF-1 receptor and the epidermal growth factor receptor (EGFR), or heterodimerize with the LHR. Interactions with these other receptors alter or augment signaling pathways, but conditions under which these interactions occur *in vivo* are not clear. For example, FSHR activated PKA promotes inactivation of the MAPK phosphatase (MKP) dual specificity phosphatase DUSP6 which in turn elicits higher ERK phosphorylation by the EGFR. Crosstalk between LH and FSH receptors may be important for reproductive function, given that during the last stages of follicle development prior to ovulation, both receptors coexist in granulosa cells. FSHR primarily activates the Gs/cAMP/PKA pathway leading to FSH-responsive gene induction and sex steroid production. However, although this pathway canonically terminates in the activation of CREB transcription factor, FSH-responsive genes in humans are enriched in binding motifs for the GATA factors, and not CREB. FSHR also activates several other signaling pathways via interaction with other G proteins, at least under certain conditions. FSHR stimulates Ca2+ mobilization in both granulosa and Sertoli cells via a variety of mechanisms. Besides possible interaction with Gq, since GPCRs can be promiscuous, APPL1 adaptor protein interacting with FSHR leads to calcium mobilization and to the activation of the protein kinase B/AKT anti-apoptotic pathway. Alternatively, cAMP activates not only PKA, but EPAC as well, which leads to the activation of PKB/AKT and MAPK pathways. Activation of PKB/AKT is involved in proliferative pathways in both Sertoli and granulosa cells and in FSH-mediated protection of granulosa cells from atresia. Subsequent to G protein activation, FSHR itself is phosphorylated by GRKs at the intracellular tail, triggering association with β-arrestins that also leads to ERK1/2 MAPK activation. ERK-activated mTOR pathway can induce the expression of FSH differentiative target genes, such as LHR, inhibin-α, and Cyp19 aromatase. Most of these pathways were identified using *ex vivo* cells, cell lines, or reconstituted heterologous cells, while

in vivo confirmation of their roles is unresolved, primarily owing to the lack of mouse models allowing cell-specific deletions. Greater understanding of FSH-mediated signaling network is of high physiological and clinical importance due to the crucial role for FSH in regulating mammalian reproduction.

Elucidation of FSH signaling pathways could also lead to designing drug candidates that may activate selective signaling pathways in target cells. Several selective, nonpeptide, small molecules have been identified as FSH agonists (reviewed in Nataraja, et al., (12)). In addition, selective, nonpeptide antagonists to FSH receptors have been generated that inhibit ovulation in rats. Antagonists have the potential to offer a highly selective, nonsteroidal contraception methods with fewer side effects than the currently available steroid-based contraceptives. Of particular interest is potential for allosteric regulation of the receptor activity, meaning that the regulator interacts at one site to modulate interactions at a spatially distinct site of the same molecule. FSHRs can be modulated at allosteric sites that are away from the orthosteric, ligand-binging sites. This feature of FSHR signaling may allow development of ligands that specifically modulate effectors with desired effects.

FSH function in extragonadal tissues

FSHR is expressed in extragonadal tissues as well, especially in osteoclasts, adipocytes, chondrocytes, benign prostatic hyperplasia and prostate cancer, ovarian cancer and placenta. These findings imply a role for FSH in these tissues that has not been studied before, and more importantly, potential FSH effects in pathophysiology in instances with high or low FSH levels.

During reproductive aging, women experience profound reductions in bone mineral density due to increased resorption rates. This phenomenon was considered to be a result of

decreased estrogen, however new results point to the possible effects of increased FSH, which as mentioned above, is elevated in menopause. This phase of women's life is associated with weight increase and visceral adiposity, as well as dysregulated energy homeostasis. Since FSHR is expressed in osteoclasts and adipocytes, it was postulated that increased FSH may be responsible for these effects of aging (review in (13) and (14)). In bone, FSH may increase osteoclast formation, function, and survival and thus, increased FSH in peri- and post-menopausal women may contribute to bone loss, since FSH can stimulate resorption by osteoclasts. Women with premature ovarian failure exhibit elevated FSH earlier, and regardless of causes of increased FSH as discussed above, they may face not only infertility, but increased risk of osteoporosis and heart disease, which is associated with increased visceral adiposity. Therefore, it is critical to address these outstanding questions.

Furthermore, FSHR overexpression is observed in a variety of endocrine tissue cancers (reviewed in (15)). FSHR was expressed primarily on the endothelial cells in the peripheral tumor blood vessels. The available evidence indicates that epithelial FSHR promotes proliferation, migration, and invasion of ovarian, prostate, and breast cancer cells. Given the propensity for distant metastases, it is important to address a possible role of FSH in angiogenesis or metastatic potential of these tumors.

Finally, in addition to tumor endothelial cells, FSHR protein was observed in normal vascular endothelium of the fetal vasculature within the chorionic villi, in glandular epithelium of the cervix, in proliferative and secretory endometrium, in muscle fibers and stroma of the myometrium, and in the placenta in pregnancy (reviewed in (16)). FSH in umbilical vein endothelial cells and in osteoclasts acts through a shorter FSHR isoform that lacks the C-terminal domain and does not stimulate cAMP production. This may explain the inability to detect FSHR

in these tissues in earlier studies. Analyses of the wildtype and FSHR null feto-placental units revealed that the FSHR null placentas have fewer fetal vessels, revealing an important role for the FSHR in fetal vessel angiogenesis. This is reminiscent of its role in cancer angiogenesis. Normal placental development is necessary for the healthy pregnancy and impairment in placental function may result in fetal growth retardation and fetal death.

Although these reports of extragonadal function of FSH are still controversial, they warrant examination using variety of approaches, potentially tissue-specific deletion of FSHR, to answer emerging questions. Studies reviewed herein, moreover, lend support for development of FSH antagonists for a treatment of osteoporosis, obesity, or endocrine tumors, in addition to new agonists for infertility discussed above. Further investigations into functions of FSH beyond gonadal tissues is also critical to evaluate the consequence of dysregulated FSH levels.

Conclusion

Undoubtedly, we came a long way since the first evidence for the pituitary role in gonadal regulation was published in 1910. The pituitary–gonadal relationship as we know it today was described in 1930 (albeit authors called the corresponding hormones different names) and a year later gonadotropin hormones were extracted from the pituitary. Afterwards, in the mid-seventies gonadotropin hormones amino acid sequences were determined and two decades later genes cloned. Nonetheless, this commentary points to the outstanding questions that need to be answered to gain further insight in FSH function and regulation. Answers to these questions are crucial from the basic science perspective to increase our understanding of the regulation of reproductive function. Moreover, the future of infertility treatment, or other pathologies mentioned above, relies on our ability to fully elucidate the function of this hormone.

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